

Magnetically treated water on phytochemical compounds of *Rosmarinus officinalis* L.

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Abstract— Irrigation using water treated with static magnetic field (SMF) has recently been used as a strategy to stimulate the growth and development of different plant species. The aim of this study was to characterize the bioactive compounds and evaluate the anatomical structure of *Rosmarinus officinalis* L. irrigated with SMF-treated water. Results demonstrate that the treatment promoted plant growth, the number of trichomes and increased concentrations of secondary metabolites. Methanol-extracted leaves revealed that rosmarinic acid was detected in both experimental groups, without a difference in the level. Camphor, α -terpineol and verbenone were determined as the most abundant compounds present in these leaf extracts and were strongly increased in plants irrigated with SMF-treated water. Similar results were also observed for endo-borneol, bornyl acetate and β -amyrin concentrations. Taken together, these results indicate that irrigation with SMF-treated water can be used to improve the production of rosemary to obtain pharmaceutical products with an increased antioxidative activity.

Keywords— rosemary, leaves, static magnetic field, thin layer chromatography, gas chromatography.

I. INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is an aromatic plant of the Lamiaceae family that is frequently used in medicine. It is an important source of polyphenols and is known for its high antioxidative activity(Suong et al., 2011).The most abundant bioactive compounds in rosemary leaves are

phenols, monoterpenes, diterpenes and their derivatives, including carnosic acid and related stable compounds such as carnosol, rosmarinol, epirosmanol and 7-methylepirosmanol(Al-Sereiti et al., 1999). Rosemary is often used for the production of natural antioxidant extracts and is reported to have a strong therapeutic potential in the treatment and prevention of many diseases including asthma, spasmogenic disorders, liver disorders and hepatotoxicity, peptic ulcers, inflammatory diseases, ischemic heart disease, arteriosclerosis, Alzheimer and poor sperm motility(Suong et al., 2011; Fernández L.F. et al., 2014).

Rosemary naturally grows throughout Cuba and is frequently found in home gardens. Recently, it has been removed from the Cuban National Formulations of Phytopharmaceuticals due to problems associated with its cultivation. Indeed, rosemary has a poor vegetative propagation and low seed production. However, it is included in the priority list of plants for the development of Natural and Traditional Medicine in Cuba(Report to the National Commission for the Development of Traditional and Natural Medicine, 2008), emphasizing the need to develop new rosemary cultivation strategies.

Irrigation with magnetically treated water is an interesting strategy to improve rosemary production, as magnetic fields are known to strongly affect shoot growth and seed germination. Indeed, many reports have described the use of magnetically treated water and magnetic fields in agriculture. Its use has been associated with an increased

plant metabolism (photosynthesis and water uptake) and improved plant growth and production. Irrigation using water treated with a static magnetic field (SMF) has been used for the cultivation of several plant species including tomato (*Solanum lycopersicum* L.), cucumber (*Cucumis sativum* L.), rice (*Oriza sativum* L.), faba bean (*Vicia faba* L.), snow pea (*Pisum sativum* L. var. macrocarpon) and chickpea (*Cicer arietinum* L.) (Gesterberger P. et al., 1978; Gilart F. et al., 2013; Grewal H.S. et al., 2011a).

This study aimed to characterize the bioactive compounds present in *R. officinalis* L. irrigated with SMF-treated water(100-150 mT)and to evaluate resulting changes in leaf histology underfield conditions.

II. MATERIAL AND METHODS

Plant material

Rosmarinus officinalis L. plants were cultivated onan experimental plot in Santiago (Cuba) and the leaves were used to preparemethanol extracts. Voucher specimen isdeposited at the Herbarium of Biodiversity and Ecology Center (BIOECO) under accession number RB 21324.

The experiment used an external magnetizer with permanent magnets designed, built and calibrated at the National Center of Applied Electromagnetism (NCAE).Magnetic induction ranged between 100 and 150 mT(Gilart F. et al., 2013). Plants were either irrigated with SMF-treated water(hereafter referred to as SMF plants) orwater not treated with SMF (referred to as control plants). Sixty plants were included in each treatment andgrown underthese conditionsfor 180 days. Irrigation was performed twice a day for 30 minutes through an air microjet system, consistingof aKSB ITUR pump and a valve-controlled system distributor. Irrigation was carried out using jets, which were set at aflow rate between 2.54 and 2.91 m^3h^{-1} . Thewater velocityrangedbetween 1.4 and 1.6 ms^{-1} .

Scanning Electron Microscopy

The analysis of trichome morphology and numberwas performed in leaf samples fixed in formalin-acetic acid-alcohol (FAA) solution (70%)(Johansen, 1940). For scanning electron microscopy (SEM), leaves were dehydrated in a graded ethanol series, submitted to critical point drying with CO_2 (Leica EM CPD-030) and coated with a thin layer of gold (Denton Vacuum Desk IV, LLC). The samples were analyzed using a JEOL-JSM 6390 LV scanning electron microscope (Jeol USA Inc) as described byGesterberger P. et al. (1978).

Preparation of rosemary extracts

In order to prepare methanol leaf extracts, *R. officinalis* leaves were dried in an oven at 40°C for 72 h. Subsequently, 3 g of dried leaf sample was macerated in100 mL of methanol for 4 to 6 h in a Soxhlet device. The extract volume was then reduced to 10 mL in a Büchi rotoevaporator and centrifuged for 3 min at 3000 x g.The supernatant was filtered through a Whatman paper (GF/A, 110 mm)and stored at 4°C until further analysis.

Gas Chromatography-Mass Spectrometry (GC-MS)

Methanol extracts were analysed usinga GC-MS system (Agilent7890A/5975C GC-MS System) equipped with a JWMS5capillary column (Agilent Technologies;30mx 25mm x 0.25 μm). The chromatographic conditions used is shown in Table 1.

Table.1: Chromatographic conditions used for GC-MS analysis of methanol extracts of *Rosmarinus officinalis* L. leaves

Parameters	Conditions
Helium carrier gas flux	1 mL min^{-1}
Injection volume	1 μL
Injector temperature	270°C
Source temperature	290°C
Interface temperature	230°C
Column temperature	60-290°C (5°C/min)
Injector	automatic
Flux range	0,95 mL min^{-1}
Massspectra	70 eV
Splitratio	1:40

Chromatograms were analyzed usingthe Automated Mass Spectral Deconvolution and Identification System(AMDIS)(MSChem) and the resulting spectra were compared tothe NIST/EPA/NIH Mass Spectral Library 2011 (National Institute of Standards and Technology, Standard Reference Mass Spectra Database, USA).

Thin Layer Chromatography/High Performance ThinLayer Chromatography (TLC/ HPTLC)

Methanol extracts were also analysedusing a CAMAG TLC/HPTLC system equipped with a Linomat V applicator, a TLC scanner 3 and a 12 bit CCD camera for photo documentation, controlled by WinCATS-4 software.A sample volume of 10 μL was spotted in 5 mm bands on a precoated silica gel glass plate (20 cm x10 cm)usinga CAMAG microliter syringe. For the development of the plate, the mobile phase solvent system consisted of toluene,

ethyl acetate and formic acid (70:20:10). To visualize the components, plates were first sprayed with vanillin in ethanol (1%) and then with a solution of sulfuric acid in ethanol (10%). The plate was heated to 110°C for 5 min and then analyzed under white light for the evaluation of terpenoids and phenylpropanoids. Alternatively, plates were analyzed using natural product reagent. In this case, they were first sprayed with 1% methanolic diphenyl boric acid-b-ethylamine ester, followed by 5% ethanolic polyethylene glycol-4000 and then evaluated under UV light (365nm) for the detection of rosmarinic acid. For each of the bands observed, the retention factor (R_f) was calculated as the ratio between the migration distance of the band and the migration distance of the solvent.

III. RESULTS AND DISCUSSION

Trichomes in SMF plants

Glandular structures are known as primary sites of secondary metabolite biosynthesis, secretion and storage, and generally consist of either simple subcutaneous glands or trichomes. Plants of the *Lamiaceae* family present both capitate and peltate glandular trichomes. Both have the same basic morphology, consisting of a basal region, a stalk and a head. Whereas capitate glandular trichomes are formed by a head with one secretory cell and a stalk containing two cells, the peltate type consists of a head with eight secretory cells, one basal epidermal cell, and a wide unicellular stalk (Boix et al., 2011; Fahn, 1979). In *R. officinalis*, non-glandular trichomes are present on the veins and leaf margins and are diverse in morphology, anatomy and microstructure. Basically, they are classified according to their morphology. They can be either unicellular or multicellular, and unbranched or branched (Marin et al., 2006; Werker et al., 1985).

Results of this study show that glandular as well as non-glandular trichomes were observed in plants subjected to both irrigation treatments, although non-glandular trichomes were more numerous (Figure 1). The number of both trichome types on the abaxial leaf surface was higher in SMF plants (Figure 1C-D) as compared to control plants (Figure 1A-B). Leaves of control plants contained approximately six peltate trichomes per mm², whereas those of SMF plants contained 16 per mm² on average. The trichomes are primary sites for biosynthesis, secretion and storage of secondary metabolites (Taiz et al., 2015). Greater efficiency could then be expected in the production of bioactive compounds for the treated plants, with respect to the control in the relation structure.

However, irrigation with SMF-treated water did not affect trichome structure, which is characterized by a prominent expandable cuticular layer (Boix et al., 2011).

Metabolites in SMF plants

The metabolites identified in methanol extracts of *R. officinalis* control plants and SMF plants are listed in Table 2. Results show that irrigation with SMF-treated water strongly increased the levels of terpenoids including the bicyclic monoterpenes camphor, endo-borneol and bornyl acetate and the triterpene β-amyrin. Furthermore, terpenoids were the major class of bioactive compounds present in SMF plants (Table 2). Monoterpene are produced in glandular trichomes, found on both leaf sides of *R. officinalis* (Boix et al., 2011). Therefore, the increased monoterpene levels in leaves of SMF plants are possibly related to their higher number of glandular trichomes (Figure 1).

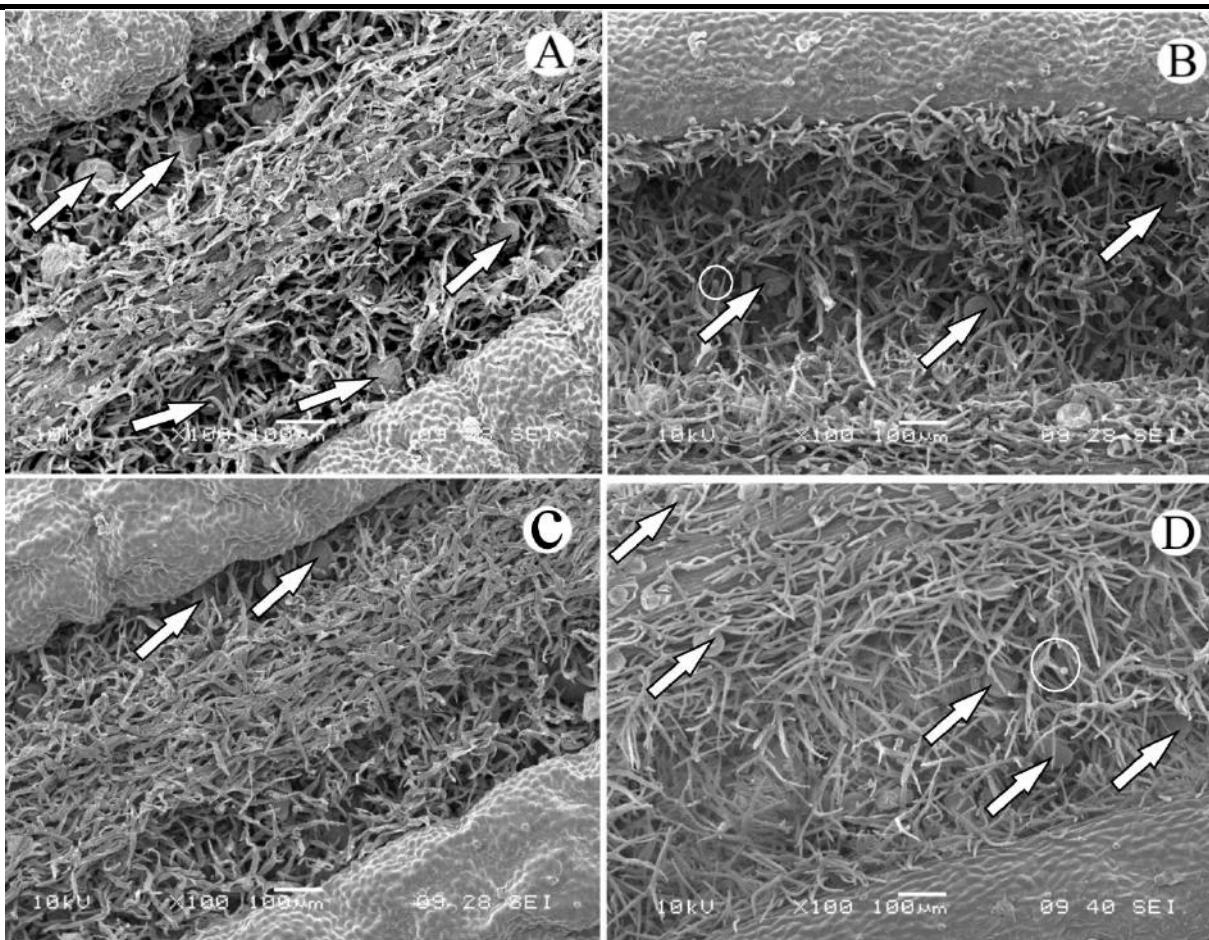


Fig.1: Scanning Electron Microscopic images of the abaxial epidermis of *Rosmarinus officinalis* L. leaves from control plants (A-B) and SMF plants (C-D). Circle: capitate glandular trichome. Arrow: peltate glandular trichome. Scale bars: 100 µm.

Table.2: Metabolites detected using GC-MS in methanol leaf extracts of *Rosmarinus officinalis* L. control and SMF plants.

Metabolites	RT	Control	SMF
		Relative area %	
camphor	6.37	16.44	23.40
endo-borneol	6.64	1.91	7.00
L-alfa-terpineol	6.80	14.00	10.3
verbenone	7.00	21.84	9.00
camphene	7.55	2.10	n.d.
bornyl acetate	8.07	2.55	14.40
2-methoxy-4-vinylphenol	8.26	0.44	n.d.
caryophyllene	10.92	3.41	3.40
phytol	26.40	11.21	9.00
β-amyrin	31.01	n.d.	18.10
squalene	41.32	12.05	n.d.
α-tocopherol	51.30	13.21	5.00
Total		99.15	99.60

Control: extract obtained from plants irrigated with water not treated with a static magnetic field; SMF: extract obtained from plants irrigated with a static magnetic field; RT: Retention time, n.d.: not detectable.

The related triterpene α -amyrin was also isolated from stems and leaves of *R. officinalis* by an extraction procedure using petroleum (60~90°C) in a study by Zhou et al. (2000). The α - and β -amyrins are pentacyclic triterpenes of natural origin, isolated from various plant sources such as resin, bark, stems, leaves, roots and rhizomes. Studies have demonstrated the pharmacological effects of these compounds against inflammation, microbial, fungal and viral infections and cancer. Furthermore, amyrins are also involved in the biosynthesis of other biologically active compounds such as avenacin, centelloside, glycyrrhizine and ginsenoside (Vázquez et al., 2012). Therefore, the higher β -amyrin levels in leaves of SMF plants as compared to control plants strongly suggest a positive effect of irrigation with SMF-treated water on the therapeutic potential of *R. officinalis*.

Furthermore, camphor concentrations were also increased in methanol leaf extracts of SMF plants as compared to those of control plants (Table 2). This oxygenated monoterpene was detected as the main compound (23.2%) in essential oils of *R. officinalis* leaves, followed by 1,8 cineol (13.4%), pinene (19.7%) and verbenone (8.2%) (Boix Y. F. et al., 2011; Boix Y.F. et al., 2014). Like amyrins, camphor has many therapeutic properties. Recently, (Rašković et al., 2014) verified the high free radical scavenging activity and hepatoprotective effects of essential oils of aerial plant parts. Interestingly, one of the most abundant compounds present in these oils was camphor.

Furthermore, results of this study showed that bornyl acetate levels were strongly increased in SMF plants as compared to control plants (Table 2). Recent studies showed that bornyl acetate lowered the production of lysophosphatidylcholine (LPS)-induced proinflammatory cytokines such as TNF- α , IL-1b, and IL-6. Therefore, irrigation of rosemary with SMF-treated water could possibly increase its anti-inflammatory potential for the treatment of inflammatory processes such as rheumatoid arthritis and osteoarthritis (Yang et al., 2014).

In addition, a decreased level of vitamin E was found in SMF plants as compared to control plants (Table 2). Vitamin E is a fat-soluble compound that is mainly localized in membranes, protecting phospholipids against oxidative degeneration by reactive oxygen species (ROS). It is involved in plant protection against oxidative damage under different stress conditions including drought, atmospheric pollutants, photosensitizing fungal toxins and

chilling (Fryer, 1992). The lower concentration of this vitamin in SMF plants possibly indicates that irrigation with SMF-treated water diminishes oxidative stress.

Using TLC/HPTLC, the presence of rosmarinic acid was confirmed in methanol extracts of control plants and SMF plants (Figure 2). The presence of this compound in *R. officinalis* extracts has already been reported by (Wagner H. et al., 1996). Rosmarinic acid is an ester of caffeic acid and has antioxidant, anti-inflammatory, antibacterial, antiangiogenic, antimutagenic and antiallergenic activities (Nunes et al., 2015). In methanolic extract obtained of control and with SMF plants the rosmarinic acid level it was no difference between both conditions. Whereas rosmarinic acid was detected under both irrigation regimes, no differences were observed in the levels of this compound in plants of either group.

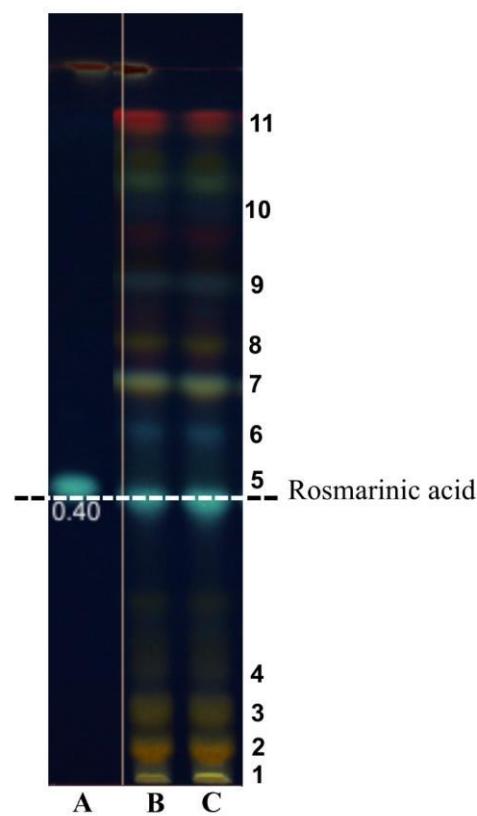


Fig.2: Thin Layer Chromatography/High Resolution Layer Chromatography (TLC/HPTLC) results showing standard rosmarinic acid ($R_f = 0.40$) (A), methanolic extracts from leaves of control (B) and SMF *Rosmarinus officinalis* L. plants (C).

Taken together, results of this study indicate that the levels of several secondary metabolites are increased in SMF plants as compared to control plants. This finding possibly explains the fact that irrigation of plants with SMF-treated

water improves plant growth. The results obtained in this study are in agreement with data available in literature. Indeed, previous studies using wheat plants demonstrated a higher total phenol content in plants irrigated with SMF-treated water as compared to control plants (Amira et al., 2010). Furthermore, it was demonstrated that irrigation with SMF-treated water leads to an increase in plant productivity and changes in water and mineral absorption (Hozayn et al., 2013; Mohamed et al., 2013). The amount of water assimilated by the plant also affects respiration and photosynthesis and the concentrations of photosynthetic pigments, phenols and indoles. Similarly, research has shown that methanolextracts of *Solanum lycopersicum* irrigated with SMF-treated water (150-300mT) had a higher phenolic content as well as a higher antioxidant activity as compared to those of control plants (Dubois et al., 2013). Interestingly, irrigation with SMF-treated water was also shown to increase the fresh and dry weight of tomato plants, implying a positive effect on plant growth (Ahmed A.M., 2013). In addition, Qados (2011) reported that irrigation with SMF-treated water significantly improved the growth and yield, as well as protein content and photosynthetic pigment levels of *Vicia faba* L. plants.

We hypothesize that the increased levels of secondary metabolites in SMF plants could be related to an effect of SMF-treated water on cell membrane characteristics, resulting in an altered cell metabolism. Indeed, (Formicchева et al. (1992)) reported that SMF-treated water significantly induced cell metabolism and mitosis in meristematic cells of pea, lentil and flax. Furthermore, gene transcription – playing an important role in regulating cellular processes – also seems to be affected by irrigation with SMF-treated water in *Pisum sativum* and *Cicer arietinum* (Grewal and Maheshwari, 2011). The effects of irrigation with magnetically treated water on plant secondary metabolite levels could also be a consequence of an influence on hormone levels, as increases in gibberellin (GA₃) and kinetin levels were observed in broad bean plants irrigated with SMF-treated water (Mohamed et al., 2013).

The effects of irrigation with SMF-treated water on these processes could be caused by changes occurring in the physical and chemical properties of water after application of a static magnetic field (Grewal et al., 2011b).

IV. CONCLUSION

Overall, results of this study indicate that irrigation with SMF-treated water could be used as a strategy to increase secondary metabolite levels in *Rosmarinus officinalis*, thereby promoting its therapeutic potential.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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